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14. ABSTRACT

A major problem in prostate cancer is finding and eliminating the non-proliferating or "quiescent" cancer cells. This is because early in prostate cancer, a small number of cancer cells metastasize to other tissues such as the bone, where they can lay dormant for years. Most chemotherapies target actively dividing cancer cells causing primary tumor shrinkage, but leave behind quiescent cancer cells which may seed new, more aggressive and chemo-resistant cancers at a later date. During this second year of funding, we have discovered that PCa cells that metastasize to the bone exhibit dramatically different cell cycle characteristics from those in the liver, suggesting signals from the bone are key to regulating PCa cell cycle and dormancy. We therefore tested signals from the marrow environment and determined how they influence the proliferation vs. quiescence decision in PCa cells. To examine how the bone marrow environment may promote PCa dormancy, we performed transcriptome analysis on mouse bone marrow cells with dormant PCa DTCs vs. recurrent PCa. We have identified secreted host marrow signals that may promote dormancy in PCa cells for study in the next funding period. We will also examine how these signals may modulate the effect of chemotherapies on PCa cell cycle regulation.

15. SUBJECT TERMS

tumor dormancy, prostate cancer cell quiescence, cancer recurrence

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INTRODUCTION:

Prostate cancer (PCa) is characterized by the early spreading of a small number of tumor cells to other tissues, termed disseminated tumor cells (DTCs). DTCs in the bone are problematic because they may lie dormant for months or even years, yet a percentage of patients will later develop recurrent cancer with significant bone metastases from these cells, which often become resistant to treatment. Understanding how DTCs reside undetected in the marrow for long periods of time and finding ways to eliminate or minimize them, is an important issue in prostate cancer research and treatment.

Hypothesis: We hypothesize that dormant DTCs enter a state of cellular quiescence in the bone marrow, which renders them insensitive to chemotherapies designed to target actively proliferating cancer cells. Recent work has revealed that quiescence may encompass multiple "depths" that impact the speed and ability of dormant cancer cells to re-enter the cell cycle. Our goal is to examine whether dormant DTCs enter into a deep quiescent state known as G0 or arrest in a quiescent but "alert" and ready to re-enter state more similar to G1 in the bone marrow and test whether the disruption of signaling from the marrow that promotes DTC quiescence may reduce tumor burden and improve treatment outcomes by sensitizing quiescent cancer cells to chemotherapies.

Aims: To address this hypothesis we are pursuing two aims: Aim 1, we develop PCa cell lines stably expressing novel cell cycle reporters which will allow us to determine the cell cycle state of DTCs during dormancy in a xenograft prostate cancer model. In Aim 2, we will target quiescence-promoting genes in these cell lines to prevent or disrupt DTC quiescence, and determine the impact on tumor dormancy, recurrence and response to current treatments.

Summary of results to date: We have successfully generated prostate cancer cell lines carrying fluorescent cell cycle sensors compatible with live imaging and flow cytometry, that together distinguish G0 and G1. We verified that these sensors accurately indicate the cell cycle state of the cells *in vitro* without disrupting their dynamics, and that these cell lines respond to signals from the bone marrow thought to promote dormancy by increasing cell cycle arrest. In our xenograft model, we have confirmed that these cell lines can form tumors and metastasize to the bone marrow. However in the process of optimizing their recovery from the marrow, we discovered that over time in the mouse the reporters become silenced. We believe this is because the reporters are integrated randomly in the genome and the extensive epigenetic remodeling that occurs during dormancy and relapse leads to their methylation and silencing. We must therefore take an alternate strategy using genetic engineering via CRISPR/Cas9 to generate cell lines where the reporters are integrated endogenously into

essential cell cycle genes to avoid epigenetic silencing. In addition we are incorporating a constitutive fluorescent nuclear marker to facilitate the recovery and imaging of these cells from the bone marrow. Despite this unexpected challenge, we have been able to perform *in vitro* and short-term *in vivo* experiments with our current cell lines. These experiments reveal that DTCs in the bone marrow accumulate in the G1-phase of the cell cycle within 48h of injection, suggesting that entry into a quiescent but "alert" state may be a very early event for DTCs in the marrow. Using *in vitro* co-culture and single cell assays we have also been able to show that signals from osteoblasts such as Gas6 promote quiescence and entry into G0, consistent with our hypothesis that the bone marrow environment promotes quiescence of DTCs over time. In the process of performing these experiments, we made the exciting discovery that two genetically identical PCa daughter cells born from the same mitosis can make different or "asymmetric" proliferation vs. quiescence decisions - for example one daughter enters G0 and remains quiescent while the other enters G1 and proceeds into the next cell cycle. We show here that the frequency of these "asymmetric" decisions can be influenced by signals from the bone and hematopoietic stem cell niche, demonstrating a mechanism by which the marrow environment influences the cell cycle status of DTCs. To identify signals from the marrow in our mouse xenograft model that may promote PCa dormancy vs. proliferation, we performed RNAseq on mouse cells isolated from bones containing DTCs or actively proliferating PCa tumors. We identified several secreted signals expressed in the mouse cells associated with DTCs and in the future, we plan to manipulate the receptors likely to receive these signals on the PCa cells to determine their roles in promoting PCa dormancy and cell cycle changes. We also plan to test whether current chemotherapies fail to eliminate, or possibly even enrich for cancer cell quiescence in the bone. In the longer term, we will also test whether disrupting genes that promote cancer cell quiescence may sensitize prostate cancer cells to chemotherapy and limit tumor recurrence.

KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

GAS6	Growth arrest specific 6
HSC	Hematopoietic stem cells
HSC Niche	Hematopoietic Stem Cell Niche
PC3	Prostate cancer cell line
PCa	Prostate Cancer
RNAseq	RNA transcriptome sequencing
G0/quiescence	A reversible quiescent or non-cycling state
FUCCI	Fluorescent ubiquitinated cell cycle indicator
FACS	Fluorescence activated cell sorting
G1	Gap1 phase of the cell cycle
S-phase	DNA synthesis phase of the cell cycle
G2	Gap2 phase of the cell cycle
Proliferation	Actively replicating and dividing cells

ACCOMPLISHMENTS: **Major goals of the project**
STATEMENT OF WORK
START DATE: Sept 15, 2016

Major Task 1: Generate prostate cancer cell lines stably expressing G0/G1 cell cycle reporters.	Months	status	**Summary of progress**
Subtask 1: Generate Lentivirus and transduce cells	1	completed	-
Subtask 2: Select stable cells lines	2	completed	-
Subtask 3: Verify reporters are correctly expressed	3	completed	-
Milestone(s) Achieved : PC-3 prostate cancer cell lines containing fluorescent cell cycle reporters		completed	PC3 labeled cell lines behave as expected in vitro. This was established in year 1
Major Task 2: Implant labeled cancer cells (or controls) into SCID mice to monitor dormancy			
Subtask 1: Insert Collagen Implants with labeled cancer cells	3wk	completed	Recovery from implants was low. We also used i.c. injection of labeled cancer cells. This improved recovery of cancer cells from the marrow
Subtask 2: Remove Collagen Implants	1		
Subtask 3: Isolate tissues and test for metastasis to bone	1-7 months	completed	We used DiD labeling of cancer cells to test for metastasis to the bone. This method is similarly sensitive as Alu PCR but allows for flow cytometry from the same sample used to test for metastasis.
Subtask 4: Isolate tissues and perform flow cytometry to identify cell cycle distributions of metastatic populations* (Timepoints throughout 1-7 months will be examined)	1-7 months	Pilot experiments complete. Optimization in progress	In short term experiments, cells that metastasized to bone (a site of tumor recurrence) were found predominantly in the G1 phase of the cell cycle, while cells that metastasized to the liver (not a site of recurrence) were in G0. (Fig3)
Subtask 5: Isolate tissues and perform immunofluorescence to identify cell cycle distributions of cancer cells in the Hematopoietic Stem cell niche* (Timepoints throughout 1-7 months will be examined)	1-7 months	Pilot experiments complete. Optimization in progress	In long-term experiments (1 month and beyond) cells injected or implanted in vivo lost expression of the cell cycle reporters (Fig 1). Clonal selection of cell lines did not resolve this problem. We therefore believe this is due to epigenetic silencing of the cell cycle reporters
Subtask 6: Isolate tissues and test for metastasis to bone using QPCR of Alu Repeats under chemotherapy treatments* (Timepoints throughout 1-7 months will be examined)	1-7 months	Pilot in progress. Optimization in progress	In order to perform this and subsequent subtasks we must generate a new cell line with cell cycle reporters integrated at endogenous gene loci, to prevent

			silencing (Fig. 2). **All other aspects of subtasks 4-7 will remain the same.**
Subtask 7: Isolate tissues and perform flow cytometry and immunofluorescence to identify cell cycle distributions of metastatic populations under chemotherapy conditions* (Timepoints throughout 1-7 months will be examined)	1-7 months	Optimization in progress	
Milestone(s) Achieved: Measurement of PC3 and C42B cell cycle dynamics during dormancy with or without chemotherapy treatment		In progress	
Major Task 3: Implant labeled cancer cells (or controls) into SCID mice to monitor tumor recurrence			
Subtask 1: Insert/Remove Collagen Implants with labeled cancer cells	1mo	Not started	
Subtask 2: Monitor tumor formation in PC3 controls labeled with Luciferase via Bioluminescent imaging with vs. without chemotherapy	7-9 months	Not started	
Subtask 3: Isolate recurrent cancers and perform flow cytometry to identify cell cycle distributions of recurrent tumor populations*	3-4 months	Not started	
Subtask 4: Isolate recurrent tumors and perform immunofluorescence to identify cell cycle distributions of cancer cells *	3-4 months	Not started	
Milestone(s) Achieved: Measurement of PC3 and C42B cell cycle dynamics during tumor recurrence- with or without chemotherapy treatment		Not started	

ACCOMPLISHMENTS & GOALS (detailed):

Major activities in year 1:

- Generate PC3 prostate cancer cell lines containing two sets of fluorescent cell cycle reporters
- Verify correct cell cycle reporter expression and cell cycle behavior of cell lines by 3 approaches - flow cytometry, live cell imaging and expression of cell cycle phase molecular markers.
- Examine the cell cycle response of cells *in vitro* to signals thought to promote tumor dormancy in bone
- Examine the cell cycle response of cells *in vitro* to chemotherapeutic agents
- Confirm that cell cycle indicator cell lines form tumors in vivo and metastasize to bone

Major activities in year 2:

- Encountered unexpected challenge that cell cycle reporters in PC3 cells implanted in mice become silenced during timecourse experiments to model tumor dormancy and recurrence. (Fig. 1)
- Developed and began an improved CRISPR/Cas9-based strategy to target reporters to endogenous gene loci in PC3 and C4-2B cells to prevent silencing and in parallel incorporate a permanent, constitutive nuclear marker to facilitate cell recovery and analysis. (Fig.2)

- Determined that cell cycle indicator cell lines that metastasize to bone vs. liver in the short term exhibit dramatically different cell cycle characteristics, suggesting signals from the bone are key to regulating PCa cell cycle and dormancy. (Fig. 3)
- Discovered using single cell tracking assays (described in year 1) that PCa cells *in vitro* undergo asymmetric cell divisions where one daughter enters quiescence while the other re-enters the cell cycle. (Fig.4)
- Determined that signals from the marrow environment (Gas6 and GM-CSF) influence the frequency of asymmetric cell divisions and the proliferation vs. quiescence decision in PCa cells providing a possible mechanism for how the bone marrow environment may promote PCa dormancy. (Fig.4)
- Performed transcriptome analysis on mouse bone marrow cells with dormant PCa DTCs vs. recurrent PCa to identify secreted host marrow signals that may promote dormancy in PCa cells. (Fig. 5)

-- PC3 cell lines carrying fluorescent cell cycle indicators lose reporter expression *in vivo*:
We used gene delivery via lentivirus, to stably integrate two different combinations of cell cycle reporters in the PC3 prostate cancer cell line (described in detail in year 1 report). Together these reporters were designed to allow us to monitor cell cycle dynamics, including G0, G1, S and G2 phases. In year 1 we successfully generated and validated *in vitro* a cell line to distinguish G0 and G1 and a second, complimentary line to monitor G0/G1, S and G2-phases. We discovered that when we performed the cell implant and metastasis assays with these cell lines as proposed in the year 1 progress report, the cells we recovered from the bone marrow lacked detectable reporter expression. This was the case for both cell lines we had established. To determine whether we were properly recovering human PCa cells from the bone marrow with our previously established technique, we performed a "pre-label" of the PCa cells with the lipophilic fluorescent dye DiD (in the far red spectrum to avoid overlap with our cell cycle reporters), and confirmed that indeed we could recover the pre-labeled cells from the mouse bone marrow. We next performed a subcutaneous (s.c.) injection of the PC3 cells carrying cell cycle reporters to confirm whether they could form tumors *in vivo* similar to the parental PC3 cell line. Indeed the PC3 cell carrying cell cycle reporters formed tumors, however when we examined the cells from those tumors by flow cytometry we found that all had lost expression of one or both reporters within a few weeks (Fig.1). Since these cells still carry the cell cycle reporters stably integrated into the genome, we suspect the reporter expression is silenced by epigenetic silencing. In support of this, we have also found that these PC3 cell lines even after re-selection and sorting by Fluorescence activated cell sorting (FACS) can lose reporter expression *in vitro* within a few passages when cultured under different grades of serum.

An improved CRISPR/Cas9-based strategy to avoid cell cycle reporter silencing and incorporate a constitutive nuclear marker
As described in the year 1 report, these reporters were integrated via lentivirus and selected by a combination of antibiotic selection and FACS. As the integration occurs randomly in the genome, there is no protection against epigenetic silencing of the reporter transgene or its promoter. To circumvent this problem, we are taking advantage of the newly developed Eflut toolkit [1] and CRISPR/Cas9 gene targeting to directly label the cell cycle proteins (p27, CDT1 and GEMININ) used in these reporters (Fig. 2) at their endogenous loci in PC3 cells. Since these are essential cell cycle genes, this should avoid issues with epigenetic silencing. In addition we will take advantage of this opportunity to stably integrate a constitutive fluorescent nuclear marker (Histone H2B-Cyan) at the human ROSA locus, which is known to work well for constitutive long-term expression [2]. The constitutive reporter will facilitate measuring recovery of the cells from the bone marrow during analysis of the cell cycle reporters by flow cytometry. Lastly, because we are using a new approach, we will also try to generate C4-2B cell lines with the same reporters to examine dormancy in an androgen-dependent context. This did not work with the previous lentivirus-based method.

PC3 cells that metastasize to bone vs. liver in the short term exhibit dramatically different cell cycle characteristics.
Although our established PC3 cell lines lose cell cycle reporter expression over time, we are able to use these cells to examine the short-term consequences of metastasis to bone vs. other sites. To examine this, we again pre-labeled cells with DiD, performed an intracardiac (i.c.) injection to mimic widespread metastasis, and 48h later isolated the human PC3 cells from the bone marrow and liver. We analyzed the reporter expression in the DiD labeled PC3 cells by flow cytometry and found that the vast majority of cells in the bone marrow exhibited

reporter activity indicating G1 phase, while cells isolated from the liver (where dormancy is rare) exhibited reporter activity indicating a mixture of G0, G1 and S/G2 and M-phases, consistent with asynchronous active proliferation.

Since nearly all of the PCa cells recovered from the bone marrow are in G1, we suggest these cells most likely have become arrested in G1. The finding that this can occur within 48h of injection is striking and suggests signals from the bone environment strongly impact the cell cycle in PCa cells. Recent work has demonstrated that quiescent stem cells may exist in two states; the traditionally quiescent G0 or a state now termed Galert which is more similar to early G1-phase where cell cycle genes are still highly expressed and cells are poised to enter the cell cycle [3]. Our new results suggests that DTCs in the bone marrow may be induced to enter a state similar to Galert with features of a G1 arrest rather than the strongly quiescent G0 state that we initially expected. This is a surprising finding and will alter our interpretation of how signals from the marrow impact the cell cycle in PCa.

-- PCa cells *in vitro* undergo asymmetric cell divisions that can be influenced by secreted signals

Using our cell tracking methodology and single-cell in vitro assays (described in year 1 report), we have discovered that PC3 cells exhibit 3 different patterns in the proliferation-quiescence decision after completing mitosis. Daughter cells from the same mitosis can make "asymmetric" decisions, where one enters the into the next cell cycle proceeding through G1 while the other enters into a prolonged quiescent G0. Alternatively both daughters can make a "symmetric" decision to enter the cell cycle proceeding through G1, or both can symmetrically enter into a quiescent state of G0. By monitoring these cell cycle decisions over time we have found that on average about 20% of PC3 cells under normal serum conditions perform asymmetric divisions where one daughter cell enters quiescent G0 (Fig.4). This is of interest since asymmetric divisions and entry into G0 are hallmarks of stem cells and may be a feature of cancer stem cells. Consistent with this idea, we find that the majority of PC3 Venus-Cherry cells that are double positive for the cancer stem cell markers CD133 and CD44 are in a quiescent G0 state (Fig. 4). This suggested to us a possible mechanism by which secreted signals in the bone marrow environment could modulate dormancy vs. cell cycle entry and recurrence. We propose that secreted signals may influence the frequency of symmetric vs. asymmetric cell cycle decisions in daughter cells. During dormancy we expect signals to lead to alterations in the rate of symmetric decisions that lead to G0 entry or G1 arrest over time to promote dormancy. Then a change in the marrow signaling environment later could promote symmetric decisions to enter into the cell cycle and proceed through G1 without arrest driving tumor recurrence. Consistent with this hypothesis, we find that a signal from osteoblasts, Gas6, which we showed previously in year 1 [4,5]can induce a G0/G1 arrest in prostate cancer cells, induces quiescence by promoting symmetric divisions where both daughter cells enter into quiescent G0. We suggest that Gas6 is just one of multiple signals in the bone marrow environment that promote cell cycle arrest and the complex signaling environment may lead to more complex outcomes such as arrest in G1 or Galert.

-- Transcriptome analysis on mouse bone marrow cells with dormant PCa DTCs vs. recurrent PCa to identify secreted host marrow signals that may promote dormancy in PCa

To identify signals from the marrow in our mouse xenograft model that may promote PCa dormancy vs. proliferation, we performed RNAseq on mouse cells isolated from bones containing DTCs or actively proliferating PCa tumors. We identified several secreted signals expressed in the mouse cells associated with DTCs and in the future, we plan to manipulate the receptors likely to receive these signals on the PCa cells to determine their roles in promoting PCa dormancy and cell cycle changes (Fig 5). For these manipulations we will using inducible shRNA constructs which we have used in this model with success previously [6].

Significant results and key outcomes

We have uncovered that PCa cells that metastasize to the bone exhibit dramatically different cell cycle characteristics than those that metastasize to the liver. Since the bone is a major site of PCa dormancy and recurrence, we hypothesize that signals from the bone marrow induce these cell cycle changes to promote dormancy. Using transcriptome analysis of mouse cells in the marrow, we have identified secreted signals that may promote dormancy in PCa. This together with our work in year 1 showing that Gas6 from osteoblasts also

promotes cell cycle arrest and increased resistance to docetaxel induced cell death, points to potential molecular combinations from the bone to promote dormancy.

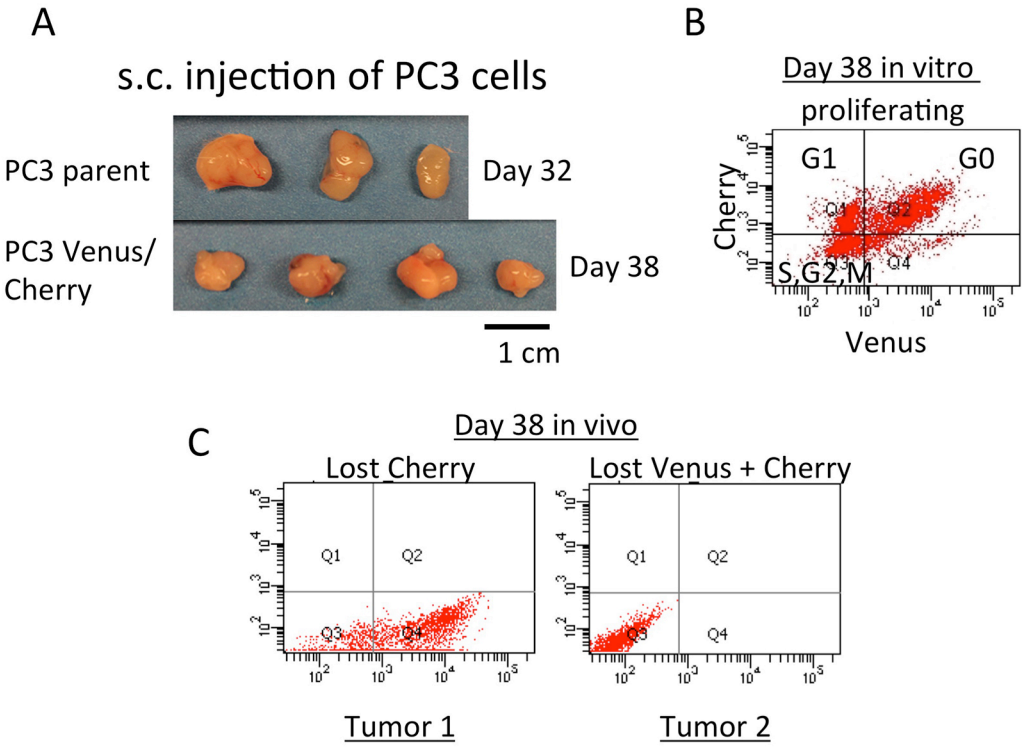
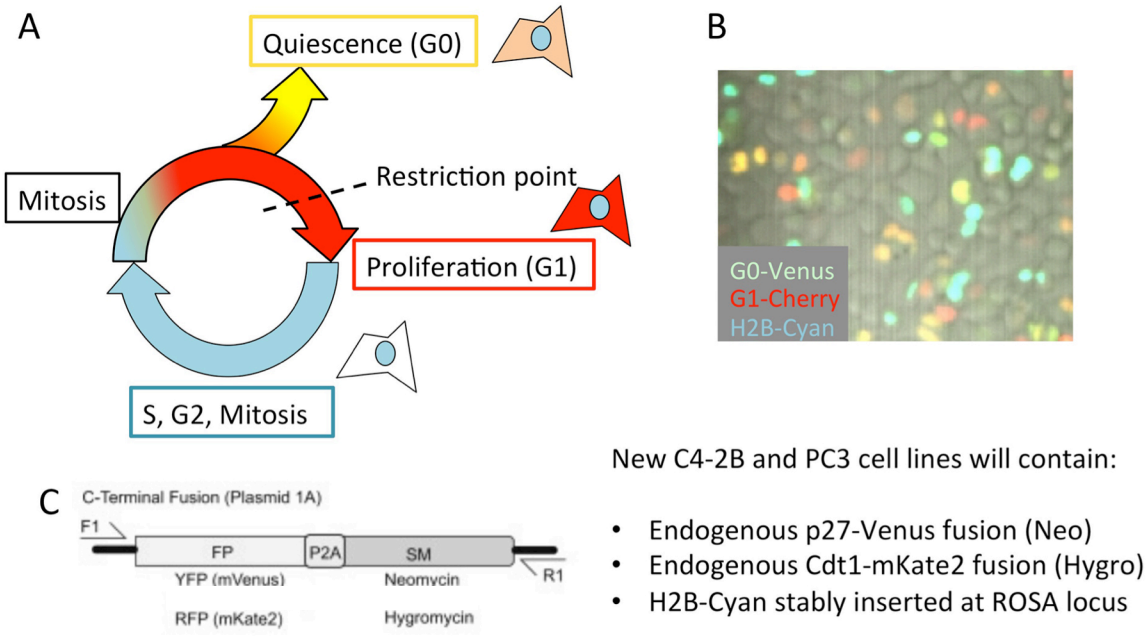


Fig.1. (A) Subcutaneous (s.c.) injection of parental PC3 or PC3 cells carrying cell cycle reporters was performed. PC3 cells from both lines form tumors *in vivo*. (B) PC3 cells carrying G0-Venus and G1-Cherry cultured *in vitro* maintain reporter expression. (C) Tumors from PC3 cells carrying cell cycle reporters lose expression of one or both reporters by day 38. Two examples are shown.



New C4-2B and PC3 cell lines will contain:

- Endogenous p27-Venus fusion (Neo)
- Endogenous Cdt1-mKate2 fusion (Hygro)
- H2B-Cyan stably inserted at ROSA locus

CFP-labeled nuclei allows long-term tracking independent of cell cycle changes and lineage tracing

Fig. 2 (A) We are using CRISPR/Cas9 gene targeting to directly label the cell cycle proteins (p27 and CDT1) at their endogenous loci in PC3 cells. In addition we will take advantage of this opportunity to stably integrate a constitutive fluorescent nuclear marker (Histone H2B-Cyan). The G01/G1 combination with nuclear Cyam will exhibit the indicated color scheme throughout the cell cycle. (B) An example of the cells we are constructing in progress (a subset of cells are Venus/Cherry/Cyan positive). (C) A diagram of the constructs from [1] we will use for integration of the reporters and selection.

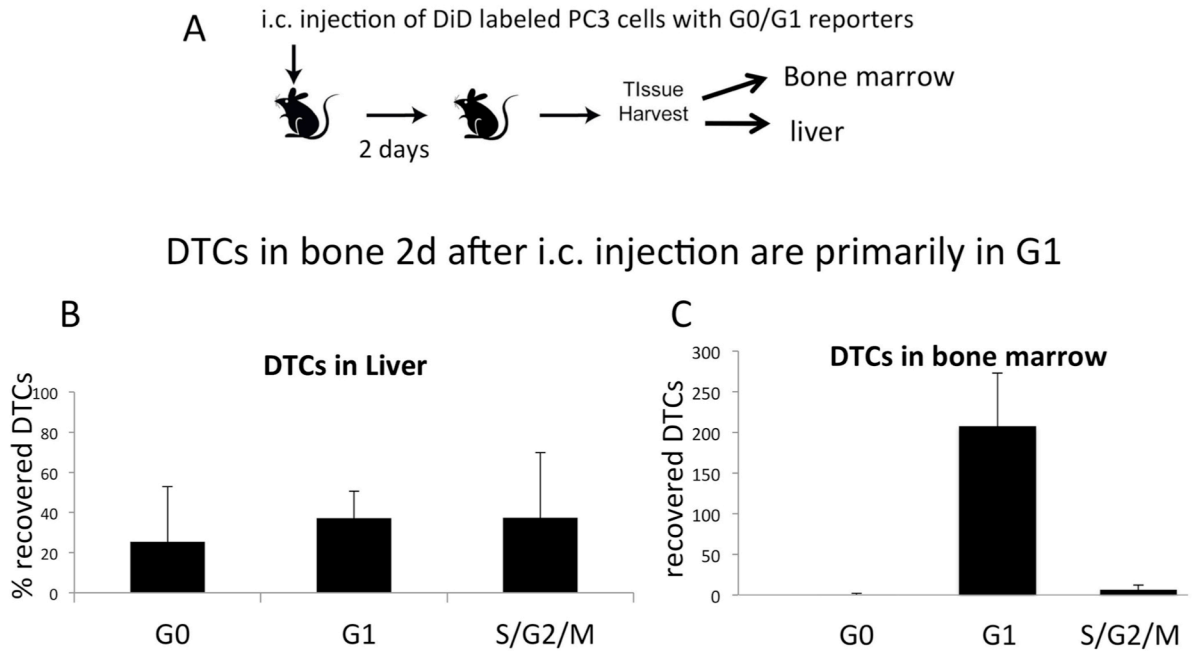


Fig. 3. (A) We performed intracardiac (i.c.) injection of PC3 cells labeled with G0/G1 cell cycle reporters and harvested liver and bone marrow 48h later to confirm metastasis. (B) Cell cycle reporter expression in the PC3 cells isolated from liver and bone was examined by flow cytometry. Cells isolated from the liver (where dormancy is rare) exhibited reporter activity indicating a mixture of G0, G1 and S/G2 and M-phases, consistent with asynchronous active proliferation. (C) Cells isolated from the bone marrow exhibit reporter activity almost exclusively indicating a G1 cell cycle arrest.

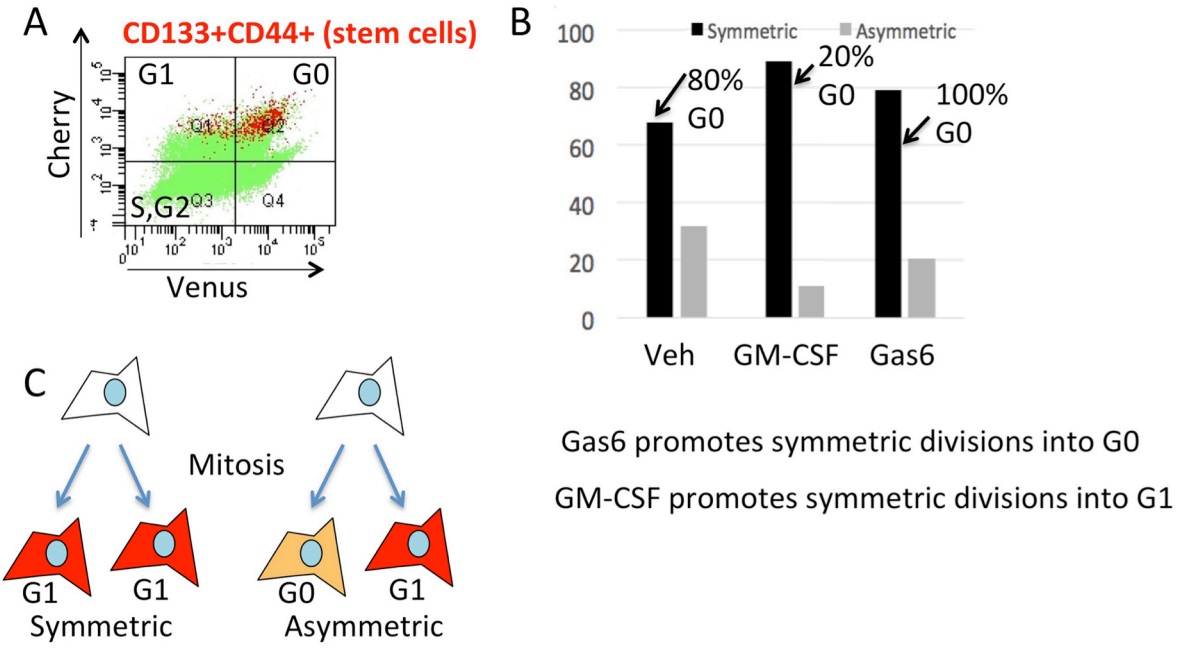
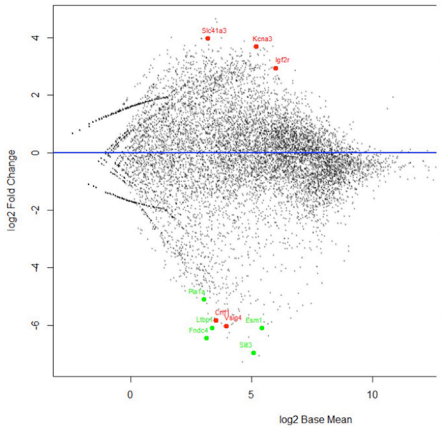


Fig. 4. (A) The majority of PC3 cells that are double positive for the cancer stem cell markers CD133 and CD44 are in a quiescent G0 state. (B) Using our cell tracking methodology and single-cell in vitro assays we found that 80% of PC3 cells under normal serum conditions perform symmetric divisions where both daughters enter G0. Under treatment with GM-CSF, symmetric divisions are increased, but only 20% of them lead to symmetric G0 entry. By contrast, treatment with Gas6 also increases symmetric divisions where all 100% of symmetric daughters enter quiescent G0. (C) A diagram showing that PC3 cells from the same mitosis can make "asymmetric" decisions, where one enters the into the next cell cycle proceeding through G1 while the other enters into a prolonged quiescent G0. Alternatively both daughters can make a "symmetric" decision to enter the cell cycle proceeding through G1, or both can symmetrically enter into a quiescent state of G0.

A

RNAseq of mouse cells associated with tumor vs. dormant PCa cells



B

Secreted host factors associated with dormancy

Host signal	PCa Receptor/effector	Possible role in quiescence
Slit3	ROBO2	Inhibit WNT signaling
Fndc4	TRPM4	Inhibit WNT signaling
Esm1	ITGB2/ITGAL	Promote adhesion to niche
Crlf1	LIFR/IL6ST	neuroendocrine differentiation/STAT signaling
Pla1a	GPR34	Promote cell survival

C Experimental design:

C4-2B/PC3 cell lines with G0/G1 reporter, constitutive nuclear reporter and inducible shRNA

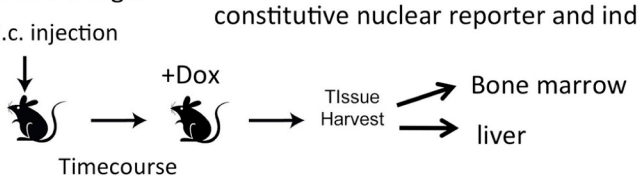


Fig. 5. (A) To identify signals from the marrow in our mouse xenograft model that may promote PCa dormancy vs. proliferation, we performed RNAseq on mouse cells isolated from bones containing DTCs or actively proliferating PCa tumors. (B) We identified several secreted signals expressed in the mouse cells associated with DTCs and in the future, we plan to manipulate the receptors likely to receive these signals on the PCa cells to determine their roles in promoting PCa dormancy and cell cycle changes. (C) The diagram describes our next experiments to use inducible shRNA to manipulate candidate dormancy receptors/effectors and examine the effects on the cell cycle of PCa cells that metastasize to the bone vs. liver.

Other achievements
We have continued to routinely verify that our cell lines are indeed PC3 by sequencing. We feel this is an important precaution, as recent work has emphasized the importance of independently verifying selected cell lines. We also routinely test for mycoplasma contamination and treat when necessary.

Stated goals not yet met

- *Monitoring tumorigenesis by timecourse in the in vivo metastatic model:* As described in detail above, we have encountered an issue with the stability of our cell cycle reporter gene expression in the xenograft model. We are using a CRISPR/Cas9 approach to address this problem and expect to be able to address this goal in the next 4 months.

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- *Measuring the effects of manipulating quiescence promoting genes:* As described above, we have identified several candidate signaling pathways that may promote quiescence in DTCs. By using the CRISPR/Cas9 approach to rebuild our cell cycle reporter cell lines with the tools described in Fig. 2, we will be able to keep one antibiotic selection marker open (Blasticidin) for integrating inducible shRNA constructs to facilitate knockdown. We plan to do this in the next 8-12 months.

Opportunities for training and professional development.
This project has provided several opportunities for training and professional development as outlined below: *Professional development activities for Dr. Buttitta under PCRP award*: Dr. Buttitta attended the 2017 Prostate Cancer Foundation Retreat in Washington D.C. This was a great networking opportunity to meet others in the prostate cancer field (which she is relatively new to) and also to learn about the newest work in various realms of prostate cancer research. In addition the work described in year one on the single cell tracking and analysis platform was presented at the AACR 2017 meeting as a poster titled "A computational and statistical approach for interpreting real-time in-vitro gene reporter data"

Dr. Taichman continues to serve as an incredibly valuable co-mentor and facilitator for Dr. Buttitta's emerging work in the prostate cancer field. Dr. Buttitta was also recently promoted to Associate Professor with tenure at the University of Michigan.

- *Collaboration between the Buttitta and Taichman Labs*: This project has provided support and a platform for continued collaboration between the Buttitta and Taichman Labs. Dr. Buttitta's lab is relatively new to the prostate cancer field, while Dr. Taichman's group has extensive experience with models for prostate cancer metastasis and recurrence. The Taichman Lab has developed protocols and techniques that have been approved by the University of Michigan University Committee on the Use and Care of Animals (UCUCA), and students and postdocs working on this project, work in close collaboration with Dr. Taichman's lab members to learn these protocols. In turn, Dr. Buttitta's lab provides the expertise and protocols for the cell cycle studies and the live cell tracking methodology. As described in further detail below, we have had three young scientists, at varying levels of experience, co-mentored by both labs through this project and an additional graduate student and junior faculty has been added this year.

1. *Co-mentorship of undergraduate student Lulia Kana*: Lulia joined the Taichman lab in 2012 where she worked on a project looking at the effects of Gas6 signaling on PC3 cells in vitro. As the collaboration led to development of new tools such as the PC3-FUCCI cell line, Lulia transitioned to being co-mentored by both labs in 2014, resulting in her work being approximately 50% in each lab. Lulia was an excellent and productive student and is now a co-author on two shared publications with the Buttitta and Taichman Labs [1, 10]. Lulia has since graduated with her BS degree and is now in medical school.
2. *Co-mentorship of graduate PhD student Dan Sun*: Dan Sun joined the Buttitta Lab as a PhD student in 2011. After completion of her first project on cell cycle regulation in *Drosophila* in early 2014, she asked to transition to working on mammalian cancer cells, since her future research interests lie in the problem of tumor dormancy. Dan has worked closely with members of the Taichman Lab to learn techniques and helped to generate the cell lines for this project as well as learning FACS protocols, cell sorting etc. This award provided tuition and stipend support for Dan in 2015. Dan was an essential

member of the team that developed the cell-tracking pipeline we use for the live image analysis of the cell cycle reporters. In July of 2016 Dan successfully defended her PhD thesis and she is now a postdoc studying cancer in Dr. Julio Aguirre-Ghiso's lab at Mt. Sinai.

3. *Co-mentorship of graduate student Ajai Pullianmackal*: Ajai joined the Buttitta lab in 2015. He is currently working on our new CRISPR/Cas9- based strategy to integrate the cell cycle reporters into PC3 and C4-2B cells. Ajai performed the single-cell experiments shown in Fig. 4 and has learned flow cytometry and cell sorting with the assistance of the Taichman Lab.
4. *Co-mentorship of Postdoctoral Fellow Kenji Yumoto*: Dr. Kenji Yumoto, a senior postdoctoral fellow in the Taichman Lab, has contributed to the training of the students on this shared project and helped to acquire a portion of the data presented here. This award has provided salary support for Dr. Yumoto since the summer of 2016. Dr. Yumoto has provided essential work on the FACS protocols for selecting cell lines, confirming the proper reporter behavior via FACS and use of the cell lines in the mouse model. Dr. Yumoto obtained most of the data shown in this progress report. As a senior scientist Dr. Yumoto has also provided valuable mentorship and training to the students contributing to this project. Dr. Yumoto is currently a co-author on two shared publications between the Buttitta and Taichman Labs [1, 10]. Our labs are located immediately across the street from each other on the University of Michigan campus, which facilitates frequent interactions between our lab members. In addition we have established formal scheduled meetings for the entire research group with both Dr. Buttitta and Dr. Taichman every other week that alternate between the School of Dentistry and Dr. Buttitta's department of Molecular, Cellular and Developmental Biology.
5. *Co-mentorship of Junior Faculty Dr. Frank Cackowski, MD PhD*: Dr. Cackowski is a Clinical Lecturer at University of Michigan working with patients and also performing research with Dr. Taichman's group. Dr. Cackowski is working to identify new molecular markers of DTCs has become an expert in working with the mouse xenograft model in the Taichman Lab to model dormancy and recurrence. The RNAseq experiment in Fig. 5 was performed by Dr. Cackowski with bioinformatic analysis performed in the Buttitta Lab and Dr. Cackowski contributed to the experiment in Fig. 3 by examining the DTCs in the mouse bone marrow.

- **How were the results disseminated to communities of interest?**

Dr. Buttitta attended the IMPaCT 2016 conference in Baltimore, MD where she presented a poster on this project to the prostate cancer research community. In addition Dr. Buttitta and collaborators presented a portion of this work as a poster at the 2017 AACR meeting. Dr. Buttitta will also present a portion of this work at the upcoming ASCB meeting cell cycle minisymposium in December 2017.

- **What do you plan to do during the next reporting period to accomplish the goals?**

In the next reporting period we will focus on obtaining data from the in vivo model using our PC3 cell lines as described in Major Tasks 2 and 3 in the SOW:

Technical challenges leading to changes in approach:

- Encountered unexpected challenge that cell cycle reporters in PC3 cells implanted in mice become silenced during timecourse experiments to model tumor dormancy and recurrence.
- Developed and began an improved CRISPR/Cas9-based strategy to target reporters to endogenous gene loci in PC3 and C4-2B cells to prevent silencing and in parallel incorporate a permanent, constitutive nuclear marker to facilitate cell recovery and analysis.
- Found that recovery from bone marrow was improved with i.c. injection compared to implants
- Found that fluorescent DiD labeling of cells aided in recovery and quantification from bone.

SOW: (changes are highlighted. **The minor technical changes in subtasks do not change the task objectives or scope**)

Major Task 2: Implant labeled cancer cells (or controls) into SCID mice to monitor dormancy			**Changes due to technical challenges and the plans for resolution**
Subtask 1: Insert **stably** labeled cancer cells by i.c. injection or implants	1-4 months	in progress	Our current cell line loses cell cycle reporter labeling in vivo after 1 month. We are generating stable lines via CRISPR/Cas9 for long-term (1-7 month) xenograft studies. Although we are generating a new cell line, *the cell cycle genes we use for reporters and other aspects of subtask 1 remain the same.*
Subtask 2: Remove Collagen Implants if used	1		
Subtask 3: Isolate tissues and test for metastasis to bone using QPCR of Alu Repeats* and detection of fluorescent labels (Timepoints throughout 1-7 months will be examined)	1-7 months	Pilot experiments complete. Requires repeat with stably labeled cell lines.	We will also use DiD labeling of cancer cells to test for metastasis to the bone. This method is similarly sensitive as Alu PCR but allows for flow cytometry from the same sample used to test for metastasis. Although we are using an additional cell label to recover metastases, *the goal (to quantify metastasis to the bone) and other aspects of subtask 3 remain the same.*
Subtask 4: Isolate tissues and perform flow cytometry to identify cell cycle distributions of metastatic populations* (Timepoints throughout 1-7 months will be examined)	1-7 months	Pilot complete. Requires repeat with stably labeled cell lines.	Subtask unchanged
Subtask 5: Isolate tissues and perform immunofluorescence to	1-7 months	Optimization in progress	Subtask unchanged

identify cell cycle distributions of cancer cells in the Hematopoietic Stem cell niche* (Timepoints throughout 1-7 months will be examined)			
Subtask 6: Isolate tissues and test for metastasis to bone using QPCR of Alu Repeats and detection of fluorescent labels under chemotherapy treatments* (Timepoints throughout 1-7 months will be examined)	1-7 months	Optimization in progress	We will also use DiD labeling of cancer cells to test for metastasis to the bone. This method is similarly sensitive as Alu PCR but allows for flow cytometry from the same sample used to test for metastasis. Although we are using an additional cell label to recover metastases, *the goal (to quantify metastasis to the bone) and other aspects of subtask 6 remain the same.*
Subtask 7: Isolate tissues and perform flow cytometry and immunofluorescence to identify cell cycle distributions of metastatic populations under chemotherapy conditions* (Timepoints throughout 1-7 months will be examined)	1-7 months	Optimization in progress	Subtask unchanged
Milestone(s) Achieved: Measurement of PC3 cell cycle dynamics during dormancy with or without chemotherapy treatment		In progress	
Major Task 3: Implant labeled cancer cells (or controls) into SCID mice to monitor tumor recurrence			
Subtask 1: Insert stably labeled cancer cells by i.c. injection or implants. Remove implants if used.	1mo	Not started	We are adding i.c. injection in addition to implants to ensure a significant fraction of animals contain cancer cells in the bone marrow. Although we are including an additional method to introduce the cancer cells, the goal (to establish metastasis to the bone) of subtask 1 remains the same.
Subtask 2: Monitor tumor formation in PC3 controls labeled with fluorescent reporters under conditions with vs. without chemotherapy	7-9 months	In vitro pilot experiments completed in year 1. In vivo work, not started	Subtask unchanged
Subtask 3: Isolate recurrent cancers and perform flow cytometry to identify cell cycle distributions of recurrent tumor populations*	3-4 months	Not started	Subtask unchanged

Subtask 4: Isolate recurrent tumors and perform immunofluorescence to identify cell cycle distributions of cancer cells *	3-4 months	Not started	Subtask unchanged
Milestone(s) Achieved: Measurement of PC3 cell cycle dynamics during tumor recurrence-with or without chemotherapy treatment		Not started	

Subtasks indicated by * will be performed in parallel, making these tasks feasible within the next year.

2. **IMPACT:**

- **Impact on the development of the principal discipline(s) of the project**

1. We are generating PC3 cell lines carrying cell cycle indicators that can be used to monitor cell cycle dynamics of cancer cells *in vitro* and *in vivo*. We expect this to be a useful tool for the scientific community.

2. We have also obtained results supporting the hypothesis that prostate cancer cells exposed to a specific signal in the bone marrow, Gas6, exhibit increased cell cycle arrest in G0/G1 phase of the cell cycle. This is significant, as we have also shown that prostate cancer cells in G0 exhibit increased resistance to Docetaxel induced cell death. Our results suggest that prostate cancer metastasis to the bone and treatment with traditional chemotherapeutic agents such as Docetaxel likely leads to a relative increase in the fraction of cancer cells in a quiescent or G0 population which may seed recurrent tumors later.

3. We have demonstrated that the bone marrow environment has dramatic effects on the cell cycle state of PCa cells and we have identified candidate signaling pathways that may mediate these effects.

- **What was the impact on other disciplines?**

The automated cell tracking method we have developed to monitor cell cycle reporters in PC3 cells can be applied to other cancer and non-cancer cell types. We expect this will be useful pipeline for other academic scientists using these cell cycle indicators in other cell types. We have a paper in revision for publication in *Scientific Reports* on this methodology, which we expect to be published in the next month. We expect our results on candidate signals from the bone marrow that promote cancer dormancy and recurrence to have impacts on the research of other cancers that also metastasize to the bone such as breast cancer.

- **What was the impact on technology transfer?**

Nothing to Report.

- **What was the impact on society beyond science and technology?**

Nothing to Report

3. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

As described in detail above, we are using a modern CRISPR/Cas9-based approach to resolve issues regarding our PCa cell lines carrying cell cycle reporters. In addition, due to problems with low metastasis and recovery of cells from the cell implant methods, we have begun to use intracardiac PC3 injections to model rapid metastasis to the bone.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

The delays in establishing stable cell lines carrying the fluorescent indicators and the difficulty in establishing the C4-2B cell line has contributed to pushing back our expected timeline for major tasks 2 and 3. We will address this by performing some subtasks for 2 and 3 in parallel (indicated by *). We plan to focus on the PC3 cell line for now, but we will also re-attempt to establish C4-2B lines in parallel.

- **Changes that had a significant impact on expenditures**

The delays in performing the *in vivo* metastasis assays with recovery from the bone marrow have shifted a portion of our anticipated animal costs for period 1 to periods 2 and 3. However we do not anticipate a change in the overall budget.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

- **Significant changes in use or care of human subjects**

None

- **Significant changes in use or care of vertebrate animals.**

The addition of an *ex vivo* bone assay is a significant change to our use of vertebrate animals. This protocol has been established and used by our collaborators the Taichman Lab, and has been approved by UCUCA.

- **Significant changes in use of biohazards and/or select agents**

None

4. **PRODUCTS:**

- **Publications, conference papers, and presentations**.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

The automated cell tracking method we have developed to monitor cell cycle reporters in PC3 cells can be applied to other cancer and non-cancer cell types. We expect this will be useful pipeline for other academic scientists using these cell cycle indicators in other cell types. We have a paper in preparation on this methodology, which we expect to be published in the next year. Once this work is published we will make all customized software scripts available to academic not-for-profit researchers as described in our data sharing plan.

5. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

Name:	*Laura Buttitta*
Project Role:	*PI*
ORCID ID	0000-0002-5064-0650
Nearest person month worked:	2.0
Contribution to Project:	*Dr. Buttitta is the directing PI for this project and is involved in all aspects of project management, student and postdoc mentorship and writing for publications and reports under this award.*
Funding	*Dr. Buttitta is supported by her nine-month appointment in the College of LS&A at*

Support:	*University of Michigan as well as a Scholar Award from the American Cancer Society and a collaborative PCF grant with Dr. Taichman*
Name:	*Russell Taichman*
Project Role:	*Senior/Key Personnel*
ORCID ID	*0000-0002-7890-0020*
Nearest person month worked:	*0.2*
Contribution to Project:	*Dr. Taichman is a senior co-mentor for Dr. Buttitta and a collaborator for this project. He assists with co-mentorship of all working on this project and provides input and advice on project management and publications.*
Funding Support:	*3P01CA093900-06 (PI: Keller) (PI Project 3: Taichman)* *The Biology of Prostate Cancer Skeletal Metastases* *Project 3: Regulation of the PCa Metastatic Phenotype by the HSC Niche* *05/1/15-04/30/20 NIH/NCI* *Targeting quiescence in prostate cancer* *PI: L. Buttitta, Collaborator: Taichman* *CDMRP, W81XWH-15-1-0413* *8/28/2015-8/30/2018* *Sympathetic Nervous System Control of Disseminated Tumor Cell (DTCs) Dormancy* *PI: Taichman* *CDMRP/ DOD, PC140665 9/1/15-8/31/18* *Mechanisms of PCa Relapse in Marrow (new project)* *Prostate Cancer Foundation (Russell Taichman, PI)* *08/22/2016 – 08/22/2018* *Effort: 1.2 Cal.*
Name:	*Kenji Yumoto*
Project Role:	*Postdoctoral Fellow*
ORCID ID	*None*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Yumoto is the senior scientist on this project and peforms the majority of the experiments and helps to provide training for students working on this project. Dr. Yumoto also contributes to project management and writing of publications. Dr. Yumoto is an expert in the mouse xenograft model and using shRNA approaches.*
Funding Support:	*Dr. Yumoto is currently supported by this award*
Name:	*Dan Sun*
Project Role:	*Graduate Student Research Assistant*
ORCID ID	*None*
Nearest person	*4*

month worked:	
Contribution to Project:	*Dr. Sun recently completed her PhD in the Buttitta Lab and worked on this project together with Dr. Yumoto.*
Funding Support:	*Dr. Sun was supported by this award as well as a Scholar Award (PI: Buttitta) from the American Cancer Society*
Name:	*Lulia Kana*
Project Role:	*Undergraduate research assistant*
ORCID ID	*None*
Nearest person month worked:	*2*
Contribution to Project:	*Ms Kana was an undergraduate co-mentored by the Taichman and Buttitta labs from 2013-2016. She worked for research credit and assisted Dr. Sun and Dr. Yumoto with experiments. She graduated in 2016.*
Funding Support:	*Ms. Kana's position was an unpaid position for research credit.*
Name:	*Frank Cackowski MD PhD*
Project Role:	*Clinical Lecturer and Research Associate*
ORCID ID	*None*
Nearest person month worked:	*0.2*
Contribution to Project:	*Dr. Cackowski is a new collaborator on this project and has worked with Dr. Yumoto on the in vivo bone metastasis*
Funding Support:	*Dr. Cackowski is supported by a collaborative PCF Challenge Grant on which he is a New Investigator*
Name:	*Ajai Pullianmackal*
Project Role:	*Graduate student Research Assistant*
ORCID ID	*None*
Nearest person month worked:	*1*
Contribution to Project:	*Mr. Pullianmackal is working with Dr. Yumoto to use CRISPR/Cas9 to re-engineer the PCa cell lines with cell cycle reporters. He is also working on optimizing the flow cytometry and single cell live imaging assays.None*
Funding Support:	*Mr. Pullianmackal is supported by a Scholar Award (PI: Buttitta) from the American Cancer Society*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

New Funding for Dr. Buttitta (PI) since Sept. 2016:
None

New Funding for Dr. Taichman (Senior/Key Personnel) since Sept. 2015:
None

6. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** Not applicable
- **QUAD CHARTS:** Not applicable

7. **APPENDICES:**

None

References

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3 *Rodgers, J. T. et al. mTORC1 controls the adaptive transition of quiescent stem cells from G0 to G(Alert). Nature **510**, 393-396, doi:10.1038/nature13255 (2014).*

4 *Lee, E. et al. Growth Arrest-Specific 6 (GAS6) Promotes Prostate Cancer Survival by G1 Arrest/S Phase Delay and Inhibition of Apoptosis During Chemotherapy in Bone Marrow. J Cell Biochem **117**, 2815-2824, doi:10.1002/jcb.25582 (2016).*

5 *Jung, Y. et al. Endogenous GAS6 and Mer receptor signaling regulate prostate cancer stem cells in bone marrow. Oncotarget **7**, 25698-25711, doi:10.18632/oncotarget.8365 (2016).*

6 *Yumoto, K. et al. Axl is required for TGF-beta2-induced dormancy of prostate cancer cells in the bone marrow. Sci Rep **6**, 36520, doi:10.1038/srep36520 (2016).*